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Presence or Absence of the Cl⁻ Channel Phospholemman in the Rectal Gland of Sharks: A Comparative Study

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Feico M.A.H. Schuurmans Stekhoven, Sjoerd E. Wendelaar Bonga, Tsung-Han Lee, and Andrew R. Bottrill (2010) Presence or absence of the Cl⁻ channel phospholemman in the rectal gland of sharks: a comparative study. *Zoological Studies* 49(3): 326-334. In this study on the presence or absence of phospholemman expression in the rectal glands of different orders of sharks, we observed the following. In the clade comprised of members of the Lamniformes and Squaliformes, the channel exists, but tends to change its primary structure, and also appears to change to a form with a higher molecular weight that, according to literature data, may be located in the cytoskeleton. In the shark orders of Orectolobiformes and Carcharhiniformes, the channel gradually disappears with *Triakis scyllium* as one of the last representatives containing a largely modified N-terminus. The final members of this clade (Carcharhinidae and Sphyrnidae) lack the channel. From these data it was concluded that the presence or absence of phospholemman is clade and family dependent, and these clades have experienced different genetic histories. <http://zoostud.sinica.edu.tw/Journals/49.3/326.pdf>

Key words: Phospholemman, Rectal gland, Shark evolution.

Phospholemman was detected as early as 1981 as a 15-kDa sarcolemmal protein upon cAMP-activated phosphorylation (Jones et al. 1981) and was demonstrated to cause hyperpolarization of activated chloride currents in *Xenopus* oocytes (Moorman et al. 1992) as well as unitary anion currents through planar lipid bilayers (Moorman et al. 1995). This characterized the protein as an anion channel although intermittent cation and anion fluxes are also registered (Kowdley et al. 1997). It appears that the protein displays several activities, as variable as modulation of the affinity of Na⁺, K⁺-ATPase for Na⁺+K⁺ (Crambert et al. 2002) as well as modulation of the Na⁺/Ca²⁺

exchange in cardiac myocytes (Zhang et al. 2003) and mediation of insulin-dependent GLUT4 translocation in adipocytes (Walaas et al. 1999). However, in marine fishes like sharks, it likely functions as an osmoregulatory anion channel (Moorman and Jones 1998).

Full-length analysis of the protein in the piked dogfish *Squalus acanthias* rectal gland was reported (Mahmoud et al. 2003). Schuurmans Stekhoven et al. (2001) published the 1st comparative study on phospholemman in rectal glands from shark species, ranging from *Squalus acanthias* (order Squaliformes and family Squalidae) to *Scyliorhinus canicula*

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(Carcharhiniformes, Scyliorhinidae). It turns out that in this range of taxa, the N-terminal amino acid sequence of this protein (13.2 kDa on the gel) declined in identity and similarity from 100% to 14%. This raised the question of whether or not phospholemman expression is present in all shark species. We therefore embarked on a comparative study that examined the expression of the phospholemman gene in rectal glands of several species of sharks collected from a wide range of geographical locations.

MATERIALS AND METHODS

Sharks, rays, and their rectal glands

A summary of the various elasmobranchs, involved in this study, with orders and families to which they belong, is given in table 1 together with the location of the catch. Rectal glands from sharks caught in the waters of western Australia were provided by Rob Lowden of Seafresh Australia (North Cairns, Queensland; representative Mr. T. Hoyer). Taiwanese sharks and rays were purchased in harbours at Taichung and Tashi, and transported to the laboratory of the Department of Life Sciences of the National Chung-Hsing University, Taichung where the rectal glands were excised. Sharks, caught at the Hatton Bank, northwestern Ireland, were worked up in the

Møreforskning Ålesund, Norway.

Weights of the rectal glands ranged between 0.21 (*Deania calcea*) and 7.3 g (*Carcharhinus plumbeus*). The glands were stored in screw-capped tubes, containing ice-cold isotonic medium as described by Skou and Esmann (1988): 25% glycerol in 50 mM triethanolamine HCl at pH 7.0. These were contained in a thermos with deeply frozen ice (-80°C), placed in a plastic foam box with cooling elements or ice (-80°C), and transported to Radboud University, Nijmegen, the Netherlands by courier who had provision for cold transportation. Upon arrival, the glands were stored at -20°C.

Isolation of the microsomal fraction

The number of glands involved in this preparation was dependent on the weight of the glands. When 1 gland weighed more than 1 g (*C. plumbeus*), 1 gland was considered sufficient; if the gland weighed less (*C. eastmani*), 6 glands were used, with an intermediate number (3) for *Hemitriakis japonica*. In a single case when the gland weighed < 1 g, we had to confine ourselves to only 1 gland (*D. calcea*) as that was all that was available. Numbers of other glands used in this study with species and number in parentheses, were: *C. sorrah* (2), *C. obscurus* (1), *Rhincodon typus* end gut (1), *S. japonicus* (2), *Orectolobus ornatus* (2), *Furgaleus macki* (2), *Sphyrna lewini*

Table 1. Shark and ray species, orders and families, used in this study with the location where caught

Order	Family	Species	Location of catch
Orectolobiformes	Orectolobidae	<i>Orectolobus ornatus</i>	W-Australian sea
	Rhincodontidae	<i>Rhincodon typus</i>	Taiwanese sea
	Hemiscylliidae	<i>Chiloscyllium plagiosum</i>	Taiwanese sea
Squaliformes	Squalidae	<i>Squalus japonicus</i>	Taiwanese sea
	Centrophoridae	<i>Deania calcea</i>	Hatton bank
		<i>Centrophorus squamosus</i>	Hatton bank
		<i>Etmopterus princeps</i>	Hatton bank
Carcharhiniformes	Somnositidae	<i>Centrocymnus coelolepis</i>	Hatton bank
		<i>Furgaleus macki</i>	W-Australian sea
		<i>Hemitriakis japonica</i>	Taiwanese sea
	Scyliorhinidae	<i>Galeus eastmani</i>	Taiwanese sea
		<i>Carcharhinus obscurus</i>	W-Australian sea
		<i>Carcharhinus plumbeus</i>	W-Australian sea
	Carcharhinidae	<i>Carcharhinus sorrah</i>	Taiwanese sea
		<i>Sphyrna lewini</i>	Taiwanese sea
Rhinobatiformes	Rhinobatidae	<i>Rhinobatos schlegelii</i>	Taiwanese sea
Myliobatiformes	Dasyatidae	<i>Dasyatis acutirostra</i>	Taiwanese sea

(2), *Etmopterus princeps* (1), *Centroscymus coelolepis* (1), *Centrophorus squamosus* (1), *Rhinobatos schlegelii* (6), and *Dasyatis acutirostra* (1).

Separation of the reddish-brown rectal gland tissue from the cream-colored coelom sac, homogenisation of the former tissue in homogenisation medium (50 mM mannitol, 2.5 mM K⁺-Hepes, and 1 mM K⁺-EDTA, at pH 7.6, containing 0.5 mM phenylmethylsulfonyl fluoride as an anti-autolysis agent), and isolation by centrifugation of the microsomal fraction by separation of the nuclei and mitochondria followed procedures described in Schuurmans Stekhoven et al. (2001). The microsomal fraction was homogenised in the above-described medium to a concentration of 10-20 mg protein/ml and stored at -20°C prior to use.

Protein determination

The protein content of 50-100 µl aliquots of the above homogenates was determined by the Lowry procedure containing sodium dodecylsulfate (SDS) as described by Peterson (1977). This was followed by 2 fold delipidation, using solubilisation in SDS (at a final concentration of 2%) and sedimentation by the addition of ice-cold CH₃OH (at a final concentration of 80%). Each sedimentation step was followed by overnight storage at -20°C and centrifugation (for 5 min at 10,000 xg and 4°C). The protein content was determined in samples (10-30 µg of protein) after the 3rd solubilisation in SDS, but before the final (3rd) addition of CH₃OH. After this 3rd delipidation procedure and drying under a stream of N₂, the pellet was dissolved in solubilisation medium (50 mM Tris-HCl (pH 6.8), 0.16 M dithiothreitol, 4% SDS, 12% glycerol, and 0.012% Br-phenolblue) to a concentration of 5 mg protein/ml.

Electrophoresis, staining, and destaining of the gel

Polyacrylamide separating gels (12%, 5 cm in height) were prepared as described by Schägger and von Jagow (1987) and were covered by 1 cm of 10% spacer then filled to the top around the comb (about 1 cm) with a 3.9% stacking gel. The cathode buffer (0.1 M Tris, 0.1 M Tricine, and 0.1% SDS; pH 8.25) and anode buffer (0.2 M Tris-HCl; pH 8.9) were as described by Schägger and von Jagow (1987).

The gels were run with 40-50 µg solubilised

microsomal protein in each lane (6 lanes in total) with prestained standards (3-188 kDa) in the 8th lane. To fully enter the proteins into the gel, 40 min at 30 V was used, whereas for full passage of the gel, an additional 2 h at 100 V was required. Faster running often produced deformation of the protein bands.

Gels were stained for 30 min at 60°C in an aqueous solution of methanol (50%), acetic acid (10%), and Coomassie brilliant blue R.250 (1.75%, w/v), and subsequently destained at the same temperature for periods of 15 min each in an aqueous solution of methanol (5%) and acetic acid (7.5%). Following destaining, gels were stored at 4°C in deionised H₂O overnight.

Mass spectrometric (MS) identification of protein bands

Coomassie brilliant blue-stained protein bands in the molecular weight (MW) range of 11-18 kDa (including the MW of phospholipase) were excised with a scalpel from the gel and transferred by forceps to 2 ml screw-capped vials containing 1.5 ml of deionized water. These were frozen at -20°C. Samples were shipped in flat-bottomed Greiner tubes of 50 ml, embedded in crushed ice at -75°C, contained in a foam box to the Protein and Nucleic Acid Chemistry Laboratory (PNAAC), University of Leicester, UK. Each sample was subjected to proteolysis using trypsin (Speicher et al. 2000), and the resulting peptides were loaded at a high flow rate onto a reverse-phase trapping column (0.3 mm i.d. × 1 mm), containing 5 µm C18 300 Å Acclaim PepMap media (Dionex, Camberley, UK) and eluted through a reverse-phase capillary column (75 µm i.d. × 150 mm) containing Jupiter Proteo 4 µm 90 Å media (Phenomenex, Macclesfield, UK) that was self-packed using a high-pressure packing device (Proxeon Biosystems, Odense, Denmark). The output from the column was directly sprayed into the nanospray ion source of a 4000 Q-Trap MS (Applied Biosystems, Warrington, UK). The analysis was carried out in the positive ion mode using data-dependent switching. De novo sequencing (<http://www.ionsource.com/tutorial/DeNovo/DeNovoTOC.htm>) was carried out on the most intense fragment ion spectra using the BioAnalyst peptide sequencing tool (Applied Biosystems). All peptide sequences obtained using this software were then manually validated. Protein identifications were made by the Basic Local Alignment and Search Tool (BLAST: www.ncbi.nlm.nih.gov/BLAST/).

ncbi.nlm.nih.gov/blast/) which searches for peptide sequences in appropriate databases.

Phylogenetic analysis

The phylogeny of sharks was built via differences in amino acid sequences of 2 fundamental mitochondrial respiratory chain components of cytochrome *b* and a_3 (subunit 1). These sequences were downloaded using the accession numbers found for the different sharks on www.pubmed.org under the protein category. The following accession numbers were applied: for cytochrome *b*: *Sphyrna lewini*, P34874; *Carcharhinus plumbeus*, P34866; *Hemirhamphys japonica*, ABD85418; *Furgaleus macki*, ABD85420; *Triakis scyllium*, ABD85422; *Scyliorhinus canicula*, NP_007626; *Lamna nasus*, AAA31884; *Carcharodon carcharias*, AAB00327; *Lamna ditropis*, AAB63135; *Squalus japonicus*, BAA08753; and *Squalus acanthias*, CAA77061. For cytochrome a_3 subunit 1, the accession numbers were: *S. lewini*, ABA10567; *Carcharhinus obscurus*, AAZ07660; *Carcharhinus sorrah*, AAZ07661; *F. macki*, AAZ07685; *Carcharodon carcharias*, ABK06081; *Isurus paucus*, ABY90951; *Isurus oxyrinchus*, ACF93964; *S. canicula*, O79403; *S. japonicus*, ABP63412; *S. acanthias*, Q9ZZ52; *Deania calcea*, AAZ07591; *Centrophorus squamosus*, AAZ07599; *Etmopterus princeps*, ABW93897; *Centroscyrnus coelolepis*, ABS31660; *Orectolobus ornatus*, AAZ07692; *Chiloscyllium plagiosum*, ABY90747; and *Rhincodon typus*, ABY91044. As outgroup representatives, 2 rays were used: *Okamejei kenojei* (Rajiformes, Rajidae) and *Mobula thurstoni* (Myliobatiformes, Mobulidae). Accession numbers for these rays for cytochrome *b* are YP_254635 and AAD12300, and for cytochrome a_3 (subunit 1) are YP_254625 and ABY90969, respectively.

Sequences were aligned using the Clustal W algorithm (available at <http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Blosum62 was used as a substitution matrix. The resulting cladogram was determined following the minimum evolution (ME) algorithm (Nei and Kumar 2000) in the program MEGA3 (Kumar et al. 2004). One shark from the Carcharhiniformes (*G. eastmani*) lacked data on the amino acid sequence of the cytochromes. This problem was resolved by comparison of 16S ribosomal RNA (accession no. AY462173) with that of 5 other species: *L. nasus*, AY830752; *S. acanthias*, AY830765; *S. canicula*, Y16067; *T. scyllium*, AY462190, and *S. lewini*, AY958661.

Since the phylogenetic tree in this case (Fig. 1B) was based on different components (proteins and ribosomal (r)RNA), we decided to show the general topology rather than the exact evolutionary distances between species.

RESULTS

Outline of the results

Data on the presence or absence of phospholemman residues are presented in table 2 in conjunction with figure 1. Full systematic names of the investigated sharks and rays are given in table 1, following the classification of Compagno (1999).

The peptide fragments, indicating the existence of phospholemman in these species, were limited to 2 to 3 types, MDPAGPDNDER (T1), FTYDYR (T2), and SNSGTATAQHLLQPGEATC (T3), corresponding to M1-R11, F12-R18, and S55-C74 (E73 deleted) of *S. acanthias* phospholemman, respectively (Schuurmans Stekhoven et al. 2001, Mahmoud et al. 2003). Up to the Lamniformes, T1-T3 were readily recognisable, but from the Lamniformes on, the N-terminal sequences significantly deviated to about 70% and 50% of the *S. acanthias* sequence (Table 2), and finally at the Scyliorhinidae, it was reduced to virtually 0% (Schuurmans Stekhoven et al. 2001). In order to establish potential evolutionary relationships, we built a phylogenetic tree of the sharks investigated in this, plus those in a previous study (Schuurmans Stekhoven et al. 2001). We were interested in determining if ancient species contained phospholemman, while those which evolved more recently lacked the channel. Since sequence information on vital proteins like cytochromes did not fully cover all species, we decided to compare the phylogenetic relationship on the basis of the cytochrome a_3 -subunit 1 (Fig. 1A) and *b* + 16S rRNA (Fig. 1B). Using the latter, allowed incorporation of the Scyliorhinidae *G. eastmani* (see "MATERIALS AND METHODS"). The 2 cladograms could not be superimposed or concatenated since in the cytochrome a_3 -1-based cladogram, the Lamniformes is adjacent to the Carcharhiniformes, whereas in the cytochrome *b*-based cladogram the Lamniformes precedes the Squaliformes. A similar situation was described before by Winchell et al. (2004) for cladograms based on large and small subunit ribosomal RNAs, simply indicating

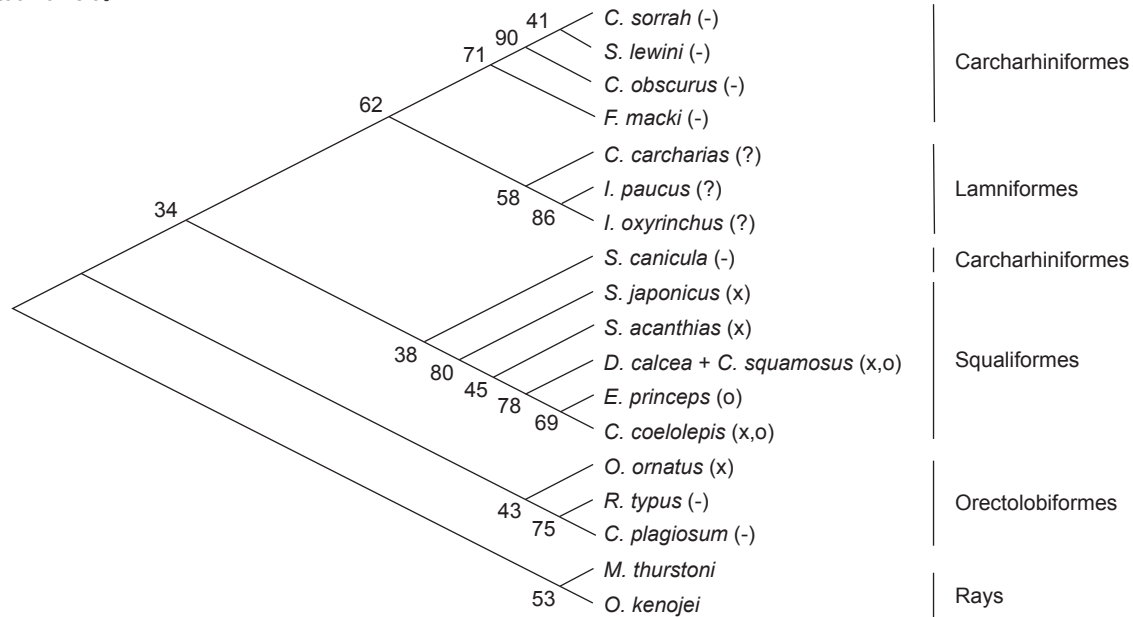
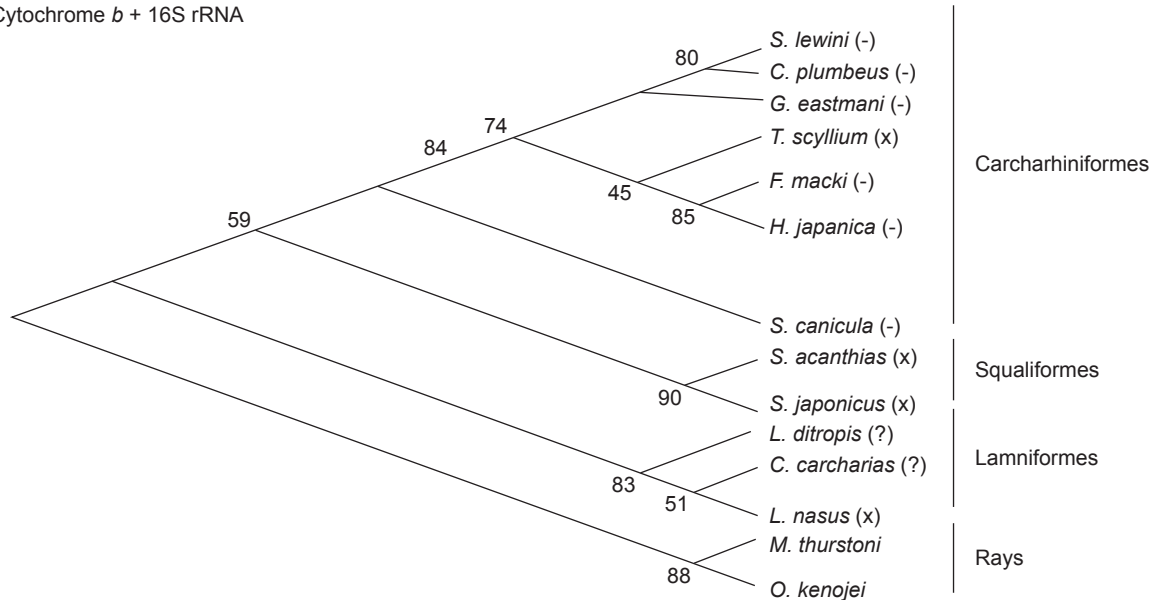
(A) Cytochrome a_3 -1(B) Cytochrome b + 16S rRNA

Fig. 1. Phylogenetic relationships within clades of shark orders with their family members, based on cytochrome a_3 , subunit 1 sequences (A), or cytochrome b + 16S rRNA sequences (B). The full systematic names of individual species, as indicated at the right-hand side of the branches, can be found in "MATERIALS AND METHODS", including order and family names (Table 1). A few other names of sharks, studied earlier (Schuurmans Stekhoven et al. 2001) are *Lamna nasus* (Lamniformes: Lamnidae), *Squalus acanthias* (Squaliformes: Squalidae), *Scyliorhinus canicula* (Carcharhiniformes: Scyliorhinidae), and *Triakis scyllium* (Carcharhiniformes: Triakidae). Since no cytochrome a_3 -1 data were available from *L. nasus*, some additional sequences of family members (*Carcharodon carcharias*, *Isurus paucus*, *I. oxyrinchus*, and *L. ditropis*) were used to construct the Lamniformes clades. Accession nos. are given in "Materials and methods". Sharks containing the phospholemmann sequences in the 12-15 kDa region, and hence probably in their cell membrane, are indicated by the letter 'x'. Phospholemmann sequences found in the 16-18 kDa region are indicated by the letter 'o', suggesting that they may occur in the cytoskeleton. The absence of phospholemmann from the rectal gland is indicated by a minus sign (-). Sharks of the family Lamnidae introduced to locate their position in the phylogenetic tree, but not analysed for the presence of phospholemmann, are indicated by a question mark (?). The 2 outgroup rays (*Okamejei kenojei* and *Mobula thurstoni*) were only used to construct the phylogenetic tree of sharks and were not analysed for the presence or absence of phospholemmann in this study.

differences in evolutionary trends among different proteins. In addition, in figure 1A, *S. canicula* belonging with *G. eastmani* to the Scyliorhinidae, clusters with the Squaliformes, whereas in figure 1B it fits into the Carcharhiniformes clade. Despite these differences, the resulting cladograms provide sufficient insight into the evolutionary trends within the different shark orders with regard to proteins such as phospholemman. Furthermore, to our knowledge, this is the 1st time that the whale shark (*R. typus*) has been given a position in the phylogenetic tree of sharks where it ranks between *O. ornatus* and *C. plagiolum* (Fig. 1A).

Phylogenetic trends of shark phospholemman

The presence of phospholemman was limited to a restricted number of hitherto investigated shark families (7 out of 12; Table 2, Fig. 1) which originate at the Orectolobidae, contain the Squaliformes and Lamniformes, and end up at *T. scyllium*, the 1st member shown of the Triakidae. Other members of this family lack the channel (Fig. 1B). The same holds for the Orectolobiformes, of which only the primary member (*O. ornatus*) contains phospholemman (Fig. 1A). In addition to certain species of shark families lacking the Cl⁻ channel, another phenomenon that can be seen from the figure is that among the Squaliformes, 2 types of phospholemman appear to exist. The 1st type, indicated by (x) in figure 1, has a molecular mass of 12-15 kDa and is basal to almost all of

the Squaliformes, except for *E. princeps*. Next to this low-molecular-mass phospholemman, another type of phospholemman of 16-17.8 kDa, indicated by (o), originates at *C. squamosus* and remains present in the subsequent members of that shark order (Fig. 1A). One of the possibilities for this phenomenon is that we are dealing with a separate form of phospholemman that is bound to the cytoskeleton instead of to the cell membrane (Kelly et al. 2004). From figure 4B of Kelly et al. (2004), a MW for this phospholemman of 17.0 kDa can be determined by interpolation, in agreement with the MW of 16-18 kDa found for the ultimate 3 members of the Squaliformes (Table 2). The fact that in *C. squamosus* the N-terminus of the 16 kDa isoform remains unchanged (Table 2) is in agreement with the finding that the cytoskeletal form is variant in its C-terminus rather than its N-terminus (Kelly et al. 2004).

DISCUSSION

Analytical procedures

In contrast to previous studies (Schuermans Stekhoven et al. 2001 2003 2004) based on blotting of gels followed by amino acid sequencing of the blot strips, the present method was based on an MS analysis of extracted tryptic fragments of these protein bands, a procedure known as *de novo* sequencing. This method has the

Table 2. Summary of phospholemman-positive shark species. The presence of phospholemman proteins and their apparent molecular weights (MWs) on the gel (kDa) in relation to order, family, and shark species are given below. Full species names are given in table 1. Species studied in Schuurmans Stekhoven et al. (2001) were *Squalus acanthias*, *Lamna nasus*, and *Triakis scyllium*

Order	Family	Species	MWs (kDa)	Ref./Type (T) ¹ or Identity (I) + Similarity (S) (%) ²
Orectolobiformes	Orectolobidae	<i>O. ornatus</i>	10-14	this paper/T2
Squaliformes	Squalidae	<i>S. japonicus</i>	13-15	this paper/T1
Squaliformes	Squalidae	<i>S. acanthias</i>	13.2	Schuermans Stekhoven et al. 2001/I+S = 100
Squaliformes	Centrophoridae	<i>D. calcea</i>	11.5-14	this paper/T2
Squaliformes	Centrophoridae	<i>C. squamosus</i>	11.5-13	this paper/T1+T2
			16	T1+T2
Squaliformes	Etmopteridae	<i>E. princeps</i>	16.2-17.0	this paper/T2
Squaliformes	Somniosidae	<i>C. coelolepis</i>	12-15	this paper/T2+T3
			17.8	T2
Lamniformes	Lamnidae	<i>L. nasus</i>	13	Schuermans Stekhoven et al. 2001/I+S = 69
Carcharhiniformes	Triakidae	<i>T. scyllium</i>	13	Schuermans Stekhoven et al. 2001/I+S = 54

¹Tryptic fragment type 1 = M₁-R₁₁; type 2 = F₁₂-R₁₈; type 3 = S₅₅-C₇₄ of *S. acanthias* phospholemman; accession nos. P82542 and CAD88978. ²N-terminal sequence identity+similarity to *S. acanthias* phospholemman; accession nos. P82542, P83009, and P83010.

advantage of all proteins within a gel band being processed simultaneously, and so the result is not dependent on the efficiency of electrotransfer. The consequence of this is that strongly basic proteins, like histones, which under blotting conditions are poorly transferred (Szewczyk and Kozloff 1985) and hence escape detection (Schuurmans Stekhoven et al. 2001), are quite easily determined using the extraction procedure. Yet, mutual interference by extracted peptides during the MS identification step is minimized by prior reverse-phase high-performance liquid chromatographic (HPLC) separation (purification) of the peptides (see "MATERIALS AND METHODS"). Further, the MS-based technique is not affected by modification of the protein N-terminus, a regular pitfall of N-terminal amino acid sequencing. Identification is based on peptides released by enzymatic digestion, so in the majority of cases, these peptides are from internal parts of the protein. In some cases, it was still possible to find N-terminal peptides, for example in the Squaliformes *S. japonicus* and *C. squamosus* (Table 2). The only disadvantage is that as leucine and isoleucine are isobaric, it is usually not possible to distinguish between these amino acids by MS. However, by BLAST searching the peptide sequences using either L or I for each instance it occurs, it is usually feasible to find the correct sequence.

Phylogenetic trends in the presence or absence of phospholemman

Our results established that the expression of phospholemman in shark rectal glands is family dependent, and not strictly related to the ancientness of the order. For instance, the Orectolobiformes, which in our phylogenetic analysis precedes the Squaliformes, contains the channel only in the Orectolobidae but not in the Rhincodontidae or Hemiscylliidae (Fig. 1A). This trend can also be seen for the Triakidae among the Carcharhiniformes (Fig. 1B). On the other hand, among the Squaliformes, the Cl⁻ channel remains in existence, although a higher-MW form has developed in subsequent stages (from *C. squamosus* on) of this clade (Fig. 1A). Its location as the cytoskeleton-bound form may be investigated by *in situ* immunology, and is not unlikely in view of the presence of other cytoskeletal components in the microsomal fraction, like β -actin, myosin, and glyceraldehyde-3-phosphate dehydrogenase (see Knull and Walsh 1992, Cao et al. 1999). Also in *L. nasus* as the last

member of the Lamniformes (Compagno 1999, Fig. 1B), the channel still exists, although in a modified form with 69% N-terminal homology compared to the Squaliform isoform (Schuurmans Stekhoven et al. 2001). Only among the most recent Carcharhiniformes, such as the Carcharhinidae and Sphyrnidae, does phospholemman appear to be systematically absent.

There may be a relationship with the abundance of Na⁺, K⁺-ATPase in the gland. Bonting (1966) found 62% lower Na⁺, K⁺-ATPase activity in the rectal gland of the Carcharhinidae *C. falciformes* compared to *S. acanthias*. A similar (67%-77%) decrease also held for skates and rays, which in our study also did not possess phospholemman. On the other hand, the number tested (2) is too small to allow firm conclusions.

The absence of phospholemman in the rectal gland might not imply its absence in other tissues like the brain, heart, or kidneys, in which we previously detected the channel in a study of *S. acanthias* (Schuurmans Stekhoven et al. 2003). This can be studied by investigating the latter tissues from a Carcharhinidae shark which apparently lacks the channel in its rectal gland.

Another cause for the "loss" of the Cl⁻-channel might be a change in the primary structure and MW so that the latter has exceeded the range within which we conducted our analyses. This may be studied by immunocomplex formation via the catch and release procedure (www.millipore.com/catalogue/item/17-500: Tech Library, User Guides: PDF 17-500 manual). In this immunoprecipitation procedure, complex formation by phospholemman is used, for instance with Na⁺, K⁺-ATPase, the latter of which is caught from the homogenate by particulate matter carrying an antibody to this enzyme.

The possibility that loss of the channel protein could have been caused by proteolysis is highly unlikely in view of the resistance to such proteolysis by phospholemman (Chen et al. 1998). Limited tryptic fragmentation of *S. acanthias* Na,K-ATPase gave rise to a 10 kDa fragment with the same N-terminal sequence as the intact 13.2 kDa phospholemman (Schuurmans Stekhoven et al. 2001). Even severe proteolysis by pronase, leading to a 9 kDa fragment, did not destroy the primary structure of the channel to an unrecognisable sequence. The resulting TYDYYRLRVVGL corresponded to T13-L24 of intact phospholemman (Schuurmans Stekhoven 1994, unpubl. data). Therefore, the absence of the channel from the rectal gland of the shark is, in our

view, a genuine and not an artificial property.

This brings us to the provisional hypothesis that in certain sharks, like those of the Carcharhinidae and Sphyrnidae, the role of the channel within the gland has become obsolete due to some unknown reason, e.g., an increase in kidney activity, thus warranting cation homeostasis.

CONCLUSIONS

Our data on the disappearance of phospholemman in certain clades of shark families inevitably led to the conclusion that this channel cannot be considered a hallmark for classification and phylogeny (see Schuurmans Stekhoven et al. 2001), since this absence pertains to different species and families in a particular clade, e.g., the Orectolobiformes and Triakidae (Fig. 1). Studies on organs other than the rectal gland are therefore necessary to reach a final conclusion. Yet, the fading away of phospholemman in rectal glands of certain shark orders might have been due to lack of selection in combination with redundancy of this channel in their gland.

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